EVALUATION OF PRESENT AND POTENTIAL PERFORMANCE OF FLOW CYTOMETRY

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Introduction

The flow cytometer measures the fluorescence and scattered light of cells and other microscopical particles as they are carried through the focus of an intense beam of excitation light by a laminar flow of water with a cross section in the order of 100 μm and a velocity around 5 m/s. Thus, the instrument can measure the cellular content of any constituent for which there exists a fluorescent dye which binds with high specificity or an antibody conjugated to a fluorochrome. The light scattering intensity is a measure of cell size. By measuring light scattering at two or more different scattering angles, usually close to 0^0 and 90^0 relative to the beam of excitation, it is possible to distinguish cells on the basis of cell shape and structure. A flow cytometer may have several fluorescence detectors for simultaneous detection of different constituents labelled with dyes emitting at different wavelengths. Thus, a typical instrument may have two detectors for light scattering and three for fluorescence.

Flow cytometers have remarkable performance. The measuring rate may exceed 10000 cells/s, the measuring precision of individual cells may be around 1 %, and the detection limit a few hundred molecules per cell, although all of this cannot be achieved at the same time. These instruments were developed primarily to measure mammalian cells, and that still is the main application. Bacteria are typically three orders of magnitude smaller in terms of volume, DNA and protein content. To perform flow cytometry on such cells the technology has to be pushed to the limits of its current performance, and some times beyond. In the following we shall discuss the principles of flow cytometry to aid the understanding of how we can fully utilise, and possibly improve, its performance.

Theoretical considerations

The sensitivity as well as the measuring precision of flow cytometers are limited by the number of photoelectrons released from the cathode of the photodetector, usually a photo multiplier tube (PMT), when a cell passes through the excitation focus. The light pulses from the cells are superimposed on a constant background of light caused primarily by scattered excitation light leaking through imperfect filters, by fluorescence from various components of the detection optics, including lenses and filters, by raman scattered light from the sheath fluid, and in some cases, e.g. by measurement of DNA, from free dye in the sample solution. Based on the fact that all emission of photons as well as photoelectrons are stochastic processes, it can be shown[1] that the fluorescence measuring precision, in terms of the signal to noise ratio of the measurement, S/N, which equals the inverse relative standard variation, cv_p, of the measurement of cells containing f fluorescent molecules, is given by:

$$(S/N)^2 = cv_p^{-2} = a\phi_e i_x f^2/(f + b) \qquad (1)$$

where b is the equivalent background, that is, the number of fluorescent molecules that would be required to produce an intensity equal to that of the background, a is a constant, ϕ_e the photoelectron quantum efficiency of the detector, and i_x the excitation intensity, i.e. the number of photons per $cm^2 \cdot s$ passing through the excitation focus. The constant, a, contains the characteristics of the fluorochrome, the detector, and the detection optics of the flow cytometer:

$$a \approx 200 \cdot \varepsilon_\lambda \cdot \phi_f (NA)^2 \cdot T \cdot l \cdot v^{-1} \qquad (2)$$

where ε_λ is the extinction coefficient of the fluorochrome at the excitation wavelength λ, ϕ_f the fluorescence quantum yield, NA the numerical aperture of the fluorescence collecting optics, T the

transmission of the detection optics, l the length of the focus in the direction of the flow, and v the flow velocity through the excitation focus.

The sensitivity in terms of the fluorescence detection limit, f_L, that is, the smallest detectable number of fluorescent molecules of a given kind per cell, is given by:

$$\text{Sensitivity} = 1/f_L = 2\phi_e a i_x /[1 + (1 + 4\phi_e a i_x b)^{1/2}] \approx [(\phi_e a i_x)/b]^{1/2} \qquad (3)$$

In practice it turns out that the term: $\phi_e a i_x b$, which is the number of photoelectrons due to background, is always significantly larger than 1, so that the approximation in Eq. 3 is generally valid. The implication of Eqs. 1 and 3 is that measuring precision and sensitivity are limited as much by the background as by the signal.

It is seen that both measuring precision and sensitivity are proportional to $i_x^{1/2}$, which is to say that to double precision and sensitivity the excitation intensity has to be quadrupled. The same improvement of sensitivity can be achieved by reducing the background to 1/4. In many cases it may be simpler and cheaper to reduce background by better filters etc. than to buy a bigger light source. Furthermore, there is a limit to the improvement that can be obtained by increasing the excitation intensity, due to photodegradation and ground state depletion of the dye. A typical dye molecule, with $\varepsilon \approx 1 \cdot 10^5$ $M^{-1}cm^{-1}$ will be excited about 200 times as it passes through a 100 μm wide 1 W laser focus with a velocity of 5 m/s. Many dyes will be degraded and left non-fluorescent after a few excitations, which is to say that the fluorescence is saturated by photobleaching. In addition to bleaching such excitation intensities may also cause ground state depletion of some dyes, that is, when intensity is high enough to keep a significant fraction of the dye molecules in an excited state at the same time. Thus, a large part of the excitation light at such intensities may be "wasted". In fact, it is worse than that. Since the background will usually not be saturated, S/N will actually decrease when i_x is increased above a certain limit. That limit depends on the dye and the flow velocity, but in general one should be cautious with laser powers above 100 mW [2]. The excitation intensity may be increased also by concentrating the light into a smaller focus. As shown below, however, this can be done only at the expense of measuring precision and reliability. Note also (Eq. 2) that the highest possible NA of the detection optics is essential for high sensitivity and precision.

Eqs. 1 - 3 apply also to the measurement of light scattering, except that ε_λ in Eq. 2 must be replaced by the scattering cross section, which is a function of the size and refractive index of the cell.

According to Eqs. 1 - 3, an alternative to increasing the excitation intensity is to reduce flow velocity, and thereby increase the total light exposure of the dye. As discussed below, however, this is done at the expense of measuring rate and precision. Reducing flow velocity also increases photobleaching, which is proportional to the total light exposure. Ground state depletion, on the other hand, is not altered.

Sensitivity

Combining Eqs. 2 and 3, we may estimate the ultimate fluorescence detection limit in flow cytometry. Assuming the use of a "bright dye", having $\varepsilon_\lambda \approx 1 \cdot 10^5$ $M^{-1}cm^{-1}$ and $\phi_f = 0{,}5$, and an instrument with v = 4 m/s, i_x = 100 mW over a focus width of 100 μm, NA = 1,3, T= 0,3, and $\phi_e = 0{,}2$, and b = 0, we find $f_L \approx 1$, which is to say that each dye molecule passing through the focus gives rise to approximately one photoelectron in the PMT. This photoelectron can be detected only provided there is no background. However, zero background is hardly feasible. In the order of 50 background photoelectrons per cell appears more realistic[1], which gives $f_L \approx 10$ dye molecules. The values used in the above estimate are in accordance with current technology. For example we have assumed $\phi_e = 0{,}2$ which is the value for todays PMTs. Furthermore, we have assumed v = 4 m/s because lower values are likely to imply photobleaching of the dye.

The detection limit of the best of current commercial instruments for FITC is a few hundred dye molecules. Such values are measured for instruments using argon lasers emitting at 488 nm, which is ideal for that dye, for example the FacScan (Becton Dickinson, San Jose, CA), the laser power of which is about 15 mW. The Bryte HS, equipped with a 100 W arc lamp, has a detection limit 2 - 3 times higher. The difference is primarily due to the fact that the excitation intensity at the peak absorption of FITC in this instrument is only about 2 mW. The detection limit of the Bryte for

dyes having their absorption maximum around one of the major mercury lines, i.e. 356, 436, 546, or 572 nm, should be significantly lower, i.e. a few hundred molecules, provided the background is similar to that observed with FITC excitation, i.e. 470 - 490 nm. The latter provision is not generally true. Thus, the background is typically decreasing with increasing excitation wavelength, due primarily to lower fluorescence of lenses and other optical components at higher wavelengths. Hence, one would expect higher detection limits (lower sensitivities) for dyes excited in the UV and deep blue and lower detection limits for dyes excited at 546 and 572 nm.

As noted above, sensitivity can be improved also by reducing flow velocity. For instruments employing a jet in air nozzle this reduction is limited by the minimum velocity required to maintain a jet. For an orifice diameter of 100 μm that minimum is about 3 m/s [4]. With closed flow cells there is no such limit, and the velocity may be reduced by as much as a factor of 1000. The real limit then is set by the photodegradation of the dye. It should be noted also that in order to take advantage of the reduced velocity the signal has to be integrated, which in practice means that the time constant of the electronics has to be increased so as to match the optical pulse length. Furthermore, it should be kept in mind that a reduction of flow velocity limits the measuring rate. To our knowledge there is no instrument on the market which facilitates a significant reduction of the flow velocity.

The sensitivity of the light scattering detection depends on background and excitation intensity accordind to Eq. 3. The background can be measured separately as explained elsewhere [1], and the detection limit, in terms of particle diameter, calculated once the excitation intensity and refractive index of the particle material are known. In practice an experimental determination can be readily done, as shown below.

Measuring rate

With the fast electronics and computers now being used in flow cytometers, the measuring rate is usually limited by the transit time, t, of the cells through the excitation focus. Cells passing simultaneously through the focus are measured as one. Assuming that the cells are randomly distributed along the flow[3], the frequency of such coincidences, ν, is given by:

$$\nu \approx f \cdot t = f \cdot l / v \quad \text{or} \quad f \approx \nu / t = \nu \cdot v / l \qquad (4)$$

where f is the average measuring rate in cells/s, v the flow velocity and l the length of the focus in the direction of the flow.

Typically, in commercial instruments $v \approx 5$ m/s, $l \approx 20$ μm. The measuring rate will depend on the rate of coincidence considered acceptable. For example, allowing a rate of coincidence: $\nu = 0{,}01$, the measuring rate becomes: $f \approx 2500$ cells/s. Reducing v (to increase sensitivity) causes a proportional reduction of the acceptable measuring rate. However, a measuring rate of a few hundred cell/s is sufficient for most purposes. Hence, the flow velocity may be reduced to around 0,5 m/s, which should triple the sensitivity of most commercial flow cytometers, provided the excitation intensity does not cause photobleaching.

The intensity profile of the excitation focus is approximately Gaussian. This implies that for all cells to be exposed to the same intensity they have to pass close to the center of the focus. For example, with a focus width (measured between the $1/e^2$ points) of 100 μm the cells have to pass within about 3 μm from the center of the focus in order to keep the exposure equal to within 1 %. To steer the cells through the focus with such precision flow cytometers are utilising the principle of "hydrodynamic focusing". In practice that is achieved by the use of a conical nozzle ending in an orifice which leads either into the open air to produce a cylindrical jet, or into a flow cell, usually with a rectangular cross section. The cell suspension is introduced through a tube having its end in the center of the flow in the conical part of the nozzle. Since the flow is laminar the sample will remain confined to the central core of the flow as it converges into the orifice. The diameter of this sample core, d, which is the precision of the cell path through the focus, is given by:

$$d = D(w/W)^{1/2} = D(w \cdot A/v)^{1/2} \qquad (5)$$

where D is the diameter of the total flow, w and W are the flow rate of the total water and the sample, respectively, and A is the cross section area of the flow through the focus. A typical value of W is 10 ml/min. If the flow diameter is 200 μm a sample flow of 10 μl/min gives $d \approx 6$ μm, which is the precision required to obtain 1 % reproducibility in the exposure in a 100 μm focus

(See above). It is seen that decreasing the flow velocity to increase sensitivity reduces the precision of the sample flow and hence of the measurement. On the other hand, reduced measuring precision is quite acceptable in many cases where biological variation is large anyway.

Flow cytometer designs

There are two basic designs on the market, employing lasers and arc lamps, respectively. Instruments with laser excitation exhibit an orthogonal configuration with the laser beam, the jet carrying the sample, and the optical axis of the fluorescence detection optics at right angles. Forward scattering is detected through optics behind a "beam dump" in the exiting laser beam, while "90^0 scatter" is detected through the fluorescence optics, and separated from the fluorescence by means of a dichroic mirror. The fluorescence may be split into two or three spectral components by dichroic mirrors and filters for simultaneous measurement of a corresponding number of chromophores. Typically a photodiode is used for the forward scatter detection, while the other detectors are PMTs. The "flow cell" may either be a jet in air or a rectangular tube. The jet in air has several significant disadvantages: The cylindrical surface of the jet gives rise to an enormous intensity of reflected light in the horizontal plane which makes it necessary to shield the detection apertures by "blocking bars" of metal or some other opaque material. These bars cause additional scattered light in the region around the focus and thereby increase the background level. Furthermore, they reduce the effective aperture of the detection optics. Since the jet in air is not compatible with immersion optics the numerical aperture of the detection optics is in practice limited to about NA = 0,6, and the effective aperture is reduced further by the "obscuration bar". Finally, the jet in air sets a lower limit to the flow velocity. Thus, a nozzle diameter of 100 μm can produce a jet only provided the velocity exceeds about 3 m/s [4]. These disadvantages are avoided with closed flow cells having a rectangular cross section, which facilitates the use of immersion detection optics with NA ≈ 1,3. Because of the approximately parallel excitation beam, the emission from non-spherical cells will depend on their orientation in the flow. This artefact may be quite significant with cells like sperm or rod-like bacteria, such as E.coli. Except for instruments with large tuneable lasers, such instruments have only a single excitation wavelength, thus limiting the selection of dyes that can be used and thereby the range of applications. Laser instruments also have the practical problem that the three orthogonal axes must be aligned to coincide with a precision of a few micrometers. This alignment may be tricky to achieve and to maintain, especially when instruments are transported or otherwise exposed to vibration.

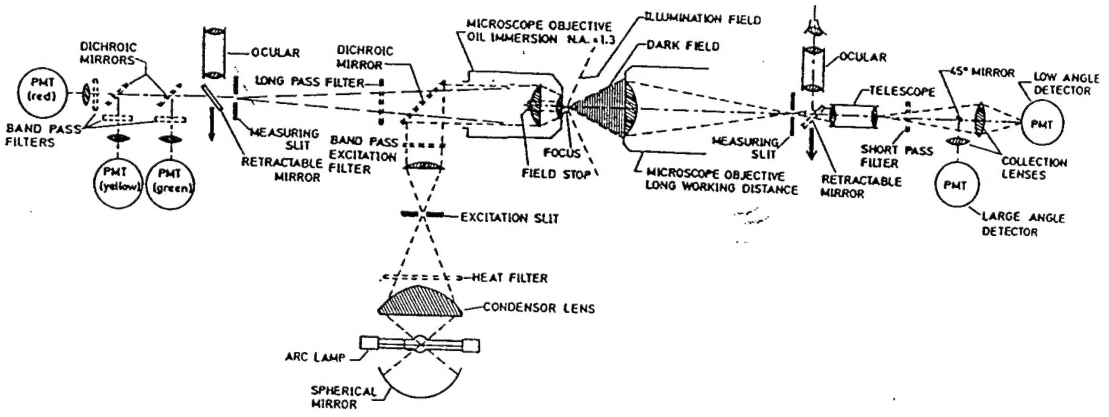

Fig. 1 Optical configuration of arc lamp-based flow cytometer with epi-illumination.

Arc lamp-based instruments employ epi-illumination through an oil immersion microscope lens in a configuration similar to that of epi-illumination fluorescence microscopes[5]. This implies that excitation and fluorescence detection are inherently confocal, which greatly simplifies alignment and makes such designs less susceptible to mechanical shock and vibration. The use of immersion optics optimises excitation intensity as well as detection efficiency. The excitation intensity attainable with

such designs is limited by the light density in the arc of the light source. High pressure mercury or xenon lamps with a power of 100 W turn out to be the best in this respect. The excitation intensities that can be achieved at the major emission lines of mercury (365, 436 and 546 nm) with a 100 W Hg lamp are 20 - 30 mW, whereas the region 470 - 490 nm, which is used for dyes like FITC, the intensity is only 2 - 3 mW [5]. This weakness can be overcome by pulsing the arc lamp. It has been found that the intensity of such lamps can be increased by a factor of 50 without adverse effects on stability and lifetime. Thus, sensitivity can be improved by a factor of 10 [6], thereby bringing the arc lamp-based flow cytometer on par with the best laser-based instruments. Alternatively, the arc lamp may be replaced or supplemented by a laser. Provided the laser beam is expanded so as to fill the aperture of the illumination optics all the advantages of the epi-illumination configuration are maintained.

A major advantage with the arc lamp is the continuos spectrum which allows the use of all dyes excitable from 350 nm and into the infrared, thereby covering the widest range of applications. The disadvantages are the relatively short life time of these lamps, i.e. 200 - 1000 hours, and a tendency to instability caused by arc movements, especially in some mercury lamps. Light scattering measurement in such instruments is provided by a dark field configuration, which facilitates separate detection of forward and large angle scattering[5,7]. An advantage of this type of instrument is the high sensitivity of the light scattering detection, which facilitates measurement of particles to below 0,2 μm (Fig. 1), as compared to about 0,5 μm for laser-based systems. This represents a 40 fold difference in the S/N. Another advantage of arc lamp-based instruments is that, due to the use of high NA lenses for both excitation and detection, they are essentially insensitive to the orientation of non-spherical cells in the flow.

Two principally different systems are employed to feed the sample into the nozzle: differential pressure and volumetric injection[5]. In the former the sample container is put under a slightly higher pressure than the sheath water reservoir by means of a pressure regulator between the two. A piece of narrow tubing (e.g. i.diam. ≈ 0,2 mm) leads from the sample into the nozzle.

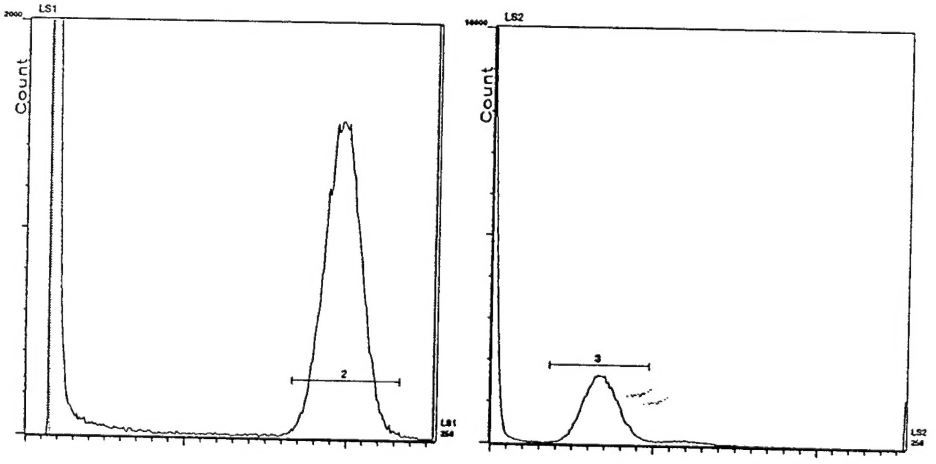

Fig. 2 *Low angle (left panel) and large angle light scattering histograms of 0,25 μm polystyrene particles illuminated through the 395 - 440 nm excitation filter of an arc lamp-based flow cytometer equipped with closed flow cell (Bryte HS, Bio-Rad, Inc. Hercules, CA). High counts in the left part of each histogram were due primarily to debris (probably silicate particles) in the sheath water. From the width of the major peak of each histogram (cv = 5,8 and 15,5 %, respectively) the detection limit of low and large angle scattering is estimated to be approximately 0,08 and 0,12 μm, respectively.*

The advantage of this system is that the sample tubing has no valves or junctions to trap cells and increase dead volume, so that sample is efficiently flushed out just by removing the sample container. The disadvantage is that the cell suspension cannot be injected at a calibrated rate, which

means that cell density cannot be determined directly. This problem is solved with volumetric sample injection where the sample is forced into the nozzle by a syringe, the piston of which is driven by a stepper motor at a calibrated rate. The disadvantage of this system is that it comprises a valve which reduces the efficiency of the flushing and thereby may increase carryover between samples.

As evident from Eqs. 1 and 3, sensitivity and measuring precision are crucially dependent on the level of background light. The background in the fluorescence channel may be reduced by the use of the highest possible quality of glass in all optical components to avoid fluorescence background, and by better optical filters to prevent scattered light from reaching the detector. Nevertheless, background cannot be completely eliminated. If nothing else, the water in the flow cell will emit Raman scattered light, that is, light scattered off the water molecules with a wavelength similar to that of fluorescence excited by the same excitation wavelength. In order to reduce this component as well as other light coming from the immediate surroundings of the illuminated volume of the sample flow, spatial filtering is employed. Thus, an aperture is situated in the image plane of the detection optics of both fluorescence and light scattering. At least in principle, this aperture should just cover the image of this volume, and thereby eliminate all light from the surroundings of the illuminated volume of the sample (Fig. 1). In practicé it must be somewhat larger to allow for less than perfect alignment of the detection optics relative to the laser focus. In laser-based instruments this aperture is a circular opening, which is called the "pinhole", whereas in some arc lamp-based instruments it is a rectangular slit covering the image of the illuminated portion of the sample flow.

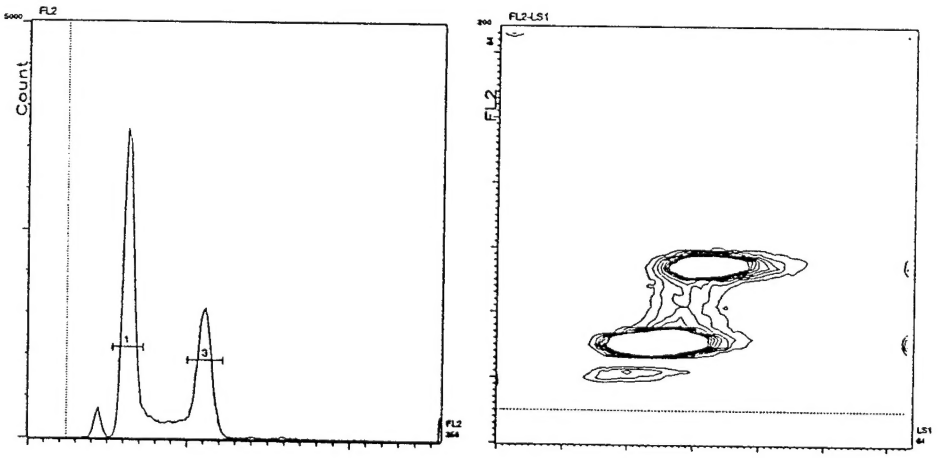

Fig. 3 Fluorescence and fluorescence versus forward light scattering histograms of wild type E. coli fixed in 70 % ethanol and stained with a combination of mithramycin and ethidium bromide which stains DNA to make it fluorescent (8). Same instrument as in Fig. 2. The cells were harvested in stationary phase of growth. The two main peaks represents cells with one and two chromosomes, respectively.

This spatial filtering eliminates out of focus light both laterally and axially, by the same principle as in confocal microscopy. Its efficiency depends on the quality of the image formed by the optics, especially with regard to contrast. Hence, microscope optics of the highest quality is important both to reduce background fluorescence from lens components and to optimise spatial filtering. Also the size of the measuring slit or "pinhole" is subject to compromise. The spatial filtering is improved by reducing the size of this aperture, but the instrument becomes increasingly dependent on perfect alignment in order to maintain the image of the cells within the aperture.

Figure 2 shows low- and large angle light scattering histograms of 0,25 μm polystyrene particles as obtained with an arc lamp-based instrument. Using Eq. 1 it can be calculated from the cv of the peaks that the detection limit for such particles is approximately 0,08 and 0,12 μm,

respectively. Recent experiments with our laboratory made prototype of the Bryte instrument indicate that the light scattering sensitivity still can be improved considerably. Thus, it appears that light scattering measurement of virus with sizes at least down to 50 nm should be feasible. Since light scattering intensity falls off with the sixth power of the linear dimensions of particles in this size range, small virus may remain outside the reach of flow cytometry for the foreseeable future. On the other hand, this strong size dependence implies highly efficient discrimination on the basis of size, and should thereby facilitate identification of virus by light scattering measurement alone.

The histograms of Fig. 3 demonstrate that the DNA and size (in terms of light scattering) of bacteria can be measured in the Bryte instrument with adequate precision. The instrument resolution at the single chromosome peak is: cv $\approx$ 4 %. Based on this number we estimate the detection limit for DNA with this type of staining to be in the order of 10 kb. New DNA specific dyes now on the market exhibit a significantly higher yield of fluorescence in the bound state, while the fluorescence of free dye, which adds to the background, is much weaker than for the dyes referred to above (Fig. 3). Furthermore, they are excited at longer wavelengths where instrument background is lower. Thus, it seems that a detection limit for DNA of about 1 kb should be within reach. In fact, measurements of DNA fragments down to a few thousand base pairs have been reported[9]. Hence, it appears that with current technology it is possible to make a flow cytometer which can measure plasmids and virus.

CONCLUSIONS

Flow cytometry of bacteria puts great demands on instrument performance due to the small size of such organisms. The fluorescence detection limit of current commercial instruments may be as low as a few hundred molecules per cell, while the size of particles down to 0,1 μm can be measured by light scattering. Further improvement may be achieved primarily by reduction of the background light caused by spurious light scattering and fluorescence. Improved sensitivity may be achievd also by pulsing of the arc lamp to increase excitation intensity, or by implementing a laser in the system. Thus, virus and plasmids down to a few hundred base pairs should be within the reach of this technology.

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